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Trait evolution and historical biogeography shape assemblages of annual killifish

Andrew J. Helmstetter[1,2] | Alexander S. T. Papadopulos[3] | Javier Igea[4] | Tom J. M. Van Dooren[5,6,7]

[1]Department of Life Sciences, Silwood Park Campus, Imperial College London, Ascot, SL5 7PY, Berkshire, UK

[2]FRB–CESAB, Montpellier, France

[3]Molecular Ecology and Fisheries Genetics Laboratory, Environment Centre Wales, School of Natural Sciences, Bangor University, Bangor, UK

[4]Department of Plant Sciences, University of Cambridge, Cambridge, UK

[5]Institute of Ecology and Environmental Sciences Paris, Sorbonne University, Paris, France

[6]CEREEP Ecotron IleDeFrance (UMS 3194), École Normale Supérieure, St-Pierre-lès-Nemours, France

[7]Naturalis Biodiversity Center, Leiden, The Netherlands

Correspondence
Andrew J. Helmstetter, Department of Life Sciences, Silwood Park Campus, Imperial College London, Ascot, SL5 7PY, Berkshire, UK.
Email: andrew.j.helmstetter@gmail.com

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Abstract

Aim: Different species assemblages of annual killifish possess replicated body size distributions yet have unique sets of species in each area of endemism. Here, we use models of trait evolution and historical biogeography to discover how size variation originated and has been restructured.

Location: South America.

Taxon: *Austrolebias* (Cyprinodontiformes).

Methods: We sampled 63 individuals from 26 *Austrolebias* species. Using phylogenetic trees (BEAST2), data on environmental variables at sampling locations and size data, we compare different models for trait evolution (SURFACE, l1OU) of body size and niche traits. We model the historical biogeography of the areas of endemism (BioGeoBEARS) and use both analyses in combination to reconstruct the history of four species assemblages.

Results: We present new phylogenetic trees for *Austrolebias* and use them to show that large size principally arose within a single area driven by a shifted selection optimum for a subset of the species in that area. We suggest that ecological interactions triggered size divergence and that this large-bodied lineage subsequently spread to two other areas. A second assemblage may have been shaped by adaptation to a new environment without an associated increase in size divergence. A third assemblage, which has the smallest size range and the most recent origin, is phylogenetically clustered, and we found no evidence of environmental filtering.

Main conclusions: Assemblage similarity in *Austrolebias* is the result of contrasting ecological, evolutionary and historical processes. Modelling trait evolution together with historical biogeography can help to disentangle the complex histories of multispecies assemblages. This approach provides context to commonly used tests investigating the role of ecological processes from phylogenetic data and generates new testable hypotheses on the processes that generated trait diversity and assemblage similarity.

KEYWORDS

annual killifish, Austrolebias, biogeography, body size, community phylogenetics, Cyprinodontiformes, phylogenetics, trait evolution

1 | INTRODUCTION

The importance of evolutionary and historical biogeographic processes in community assembly is increasingly acknowledged (Gerhold, Carlucci, Procheş, & Prinzing, 2018), but they remain relatively understudied when compared to local and recent ecological processes (Mittelbach & Schemske, 2015; Warren, Cardillo, Rosauer, & Bolnick, 2014). In community phylogenetics (Webb, Ackerly, McPeek, & Donoghue, 2002), phylogenetic reconstructions are used to characterize assemblages and to determine the ecological processes at work in them. An assemblage that is phenotypically overdispersed is usually inferred to be structured by competition while phenotypically clustered assemblages are thought be shaped by environmental filtering. However, in either case the assemblages may or may not be *phylogenetically* overdispersed (Emerson & Gillespie, 2008). Moreover, phylogenetic clustering methods are susceptible to interpreting historical effects on current geographic distributions as evidence for ecological and evolutionary processes (Warren et al., 2014).

To avoid this misinterpretation, one can model the process of trait evolution instead of interpreting patterns in observed traits. Trait evolution is an essential process in the formation of species assemblages (Kraft, Cornwell, Webb, & Ackerley, 2007; Webb et al., 2002). Often, simple evolutionary models where trait differences increase with phylogenetic distance are assumed. In reality, traits can evolve in response to the different selection regimes encountered during the history of a species, and this heterogeneity is ignored under a Brownian motion (BM) model (Cavender-Bares, Kozak, Fine, & Kembel, 2009). Trait evolution models that accommodate this determine whether species traits in an assemblage tend towards different phenotypic optima, namely by identifying selection regime shifts across a phylogeny (Butler & King, 2004). By reconstructing the history of selection regimes we can improve our understanding of the role trait evolution plays in diversification and coexistence (Oke, Rolshausen, LeBlond, & Hendry, 2017).

Historical biogeography is also an intrinsic component in the development of species assemblages, but is often neglected in empirical studies (Warren et al., 2014). The geographic and ecological shifts associated with divergence events in a phylogeny provide insights into factors shaping the diversification of the lineage. In order to incorporate the geography of speciation into phylogenetic studies, ancestral range estimation (ARE) models have often been employed. Under these models, species are assigned to predefined areas that are used to estimate the frequencies of different types of 'cladogenetic events'. In this context, founder-event speciation (i.e. long-distance dispersal followed by isolation and speciation; Matzke, 2014) lies at one end of the geographic continuum and 'within-area speciation' at the other. If we are to better understand processes that form species assemblages, more detailed joint investigations of trait evolution and biogeography along phylogenetic trees are needed.

We investigated a genus of freshwater fishes (*Austrolebias*; Costa, 1998) with naturally replicated assemblages—i.e. different assemblages with comparable size variation but different sets of related species (Figure 1)—to better understand how trait evolution and historical biogeography have shaped them. These annual killifishes live in seasonal freshwater pools and wetlands on the South American grasslands and floodplains. Eggs persist within the soil, going through diapause to survive the dry season (Wourms, 1972). *Austrolebias* are principally found in the seasonal ponds distributed throughout basins of the La Plata and Paraguay Rivers, the Negro River drainage of the Uruguay river basin and the Patos-Merin lagoon drainage system (Figure 1). These four areas of endemism (Costa, 2010) each contain a distinct assemblage and Costa (2010) hypothesized that marine transgressions during the middle and late Miocene (c. 15–11 million years) caused alternating periods of isolation and connection, driving dispersal and vicariance.

The largest *Austrolebias* species can grow to more than 150 mm in length while the smallest can be less than 25 mm when mature (Costa, 2006). Differently sized species are known to locally coexist in each area (Figure 1). Body size influences resource use in freshwater systems (Woodward & Hildrew, 2002) and stronger competition is expected between those species with similar body sizes because they use the same resources (MacArthur & Levins, 1967). Diet has already been shown to be linked to body size in *Austrolebias* (Laufer et al., 2009; Ortiz & Arim, 2016) and the largest species are known to prey upon the smaller species (Costa, 2009) indicating that differently sized species occupy distinct niches in a pond. Indeed, an ecological study within a single area found that local community structure was determined more by body size than species identity (Canavero, Hernández, Zarucki, & Arim, 2014). Considering and reconstructing body size evolution is therefore key to understanding how *Austrolebias* assemblages developed and will help to uncover how ecological interactions, such as competition and predation, have affected assemblages.

Austrolebias species may be adapted to different seasonal environments on the South American continent, which could affect the ability of a species to colonize a new area and therefore how species assemblages are formed. We refer to quantitative representations of the environment in which a species exists as its environmental niche. We applied a commonly used heuristic and studied changes in environmental niche using the same methods as for phenotypic traits (Münkemüller, Boucher, Thuiller, & Lavergne, 2015).

In this study we use models of trait evolution, environmental niches and historical biogeography together to disentangle the processes that led to the replicated body size distributions across *Austrolebias* assemblages. Niches were determined by values of environmental variables at sampling locations. Smaller scale processes, such as within-pond niche segregation (Loureiro et al., 2015), are not considered as, to date, no such data exist at the scale of this study. We tested whether inferred divergence events were associated with shifts in environmental niche traits or body size towards a new optimal value of a species relative to its ancestor. We expect that environmental niche shifts could be associated with dispersal events between areas, or might occur with dispersal within a large, heterogeneous area of endemism. To determine whether shifts coincided with migration events between areas of endemism or occurred within an area, we estimated ancestral ranges in *Austrolebias*.

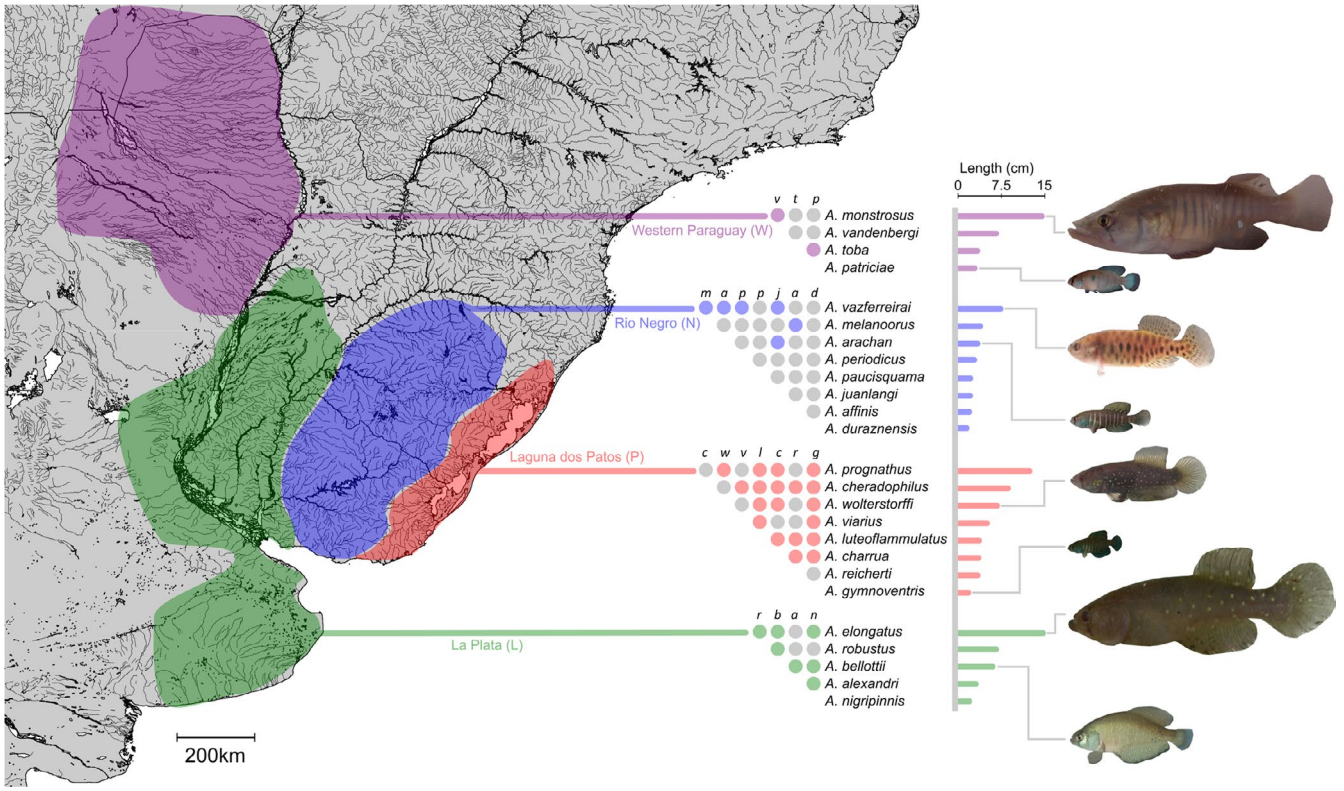

FIGURE 1 A map of eastern South America depicting the four main areas of endemism in *Austrolebias*, inferred by drawing shapes around sampling locales for species in each region. The most northern region, in purple, is the area west of the River Paraguay (W). The basin of the La Plata river and its delta are in green (L). The drainage of the Negro River, part of the River Uruguay basin is represented in blue (N). The Patos-Merin Lagoon region is highlighted in red (P). The species used in this study are shown on the right side of this figure, grouped by area. *Austrolebias apaii* and *Austrolebias cinereus* are not shown. Body size measurements are depicted as bars alongside species names. Local patterns of species co-occurrence are shown in the pairwise table to the left of species names. Coloured dots indicate that a record of a pond with both species was found or stated in Loureiro et al. (2015). Images of fishes are approximately to scale. All fish images are of males except *Austrolebias vazferreirai*

The combination of modelling shifts in body size and environmental niche traits with ancestral range estimation may shed light on how major trait variation arose within *Austrolebias* and spread to compose the current species size distributions in each area of endemism.

Here we sequence nuclear (nDNA) and mitochondrial DNA (mtDNA) markers in 26 species to reconstruct the evolutionary history of *Austrolebias* and address four main questions; (a) can we detect phylogenetic clustering or overdispersion in killifish assemblages?; (b) has body size or environmental niche convergence been important in the formation of assemblages?; (c) to what extent has diversity across lineages been shaped by divergence within-areas versus between different areas? and (d) have similar assemblages followed the same ecological and evolutionary trajectories?

2 | MATERIALS AND METHODS

2.1 | DNA sequencing

We used the Qiagen DNeasy Blood & Tissue kit to extract genomic DNA from 60 individuals of 26 *Austrolebias* species obtained from field sampling and populations maintained at CEREEP in Nemours, France. All individuals were sequenced on an ABI 3130xL Genetic Analyzer for fragments of ectoderm-neural cortex protein 1 gene (enc1), recombination activating gene 1 (rag1), SH3 and PX domain containing 3 (snx33), rhodopsin (rh1) and three fragments of 28S ribosomal DNA (28S-rRNA). Three mitochondrial genes were sequenced: 12S ribosomal DNA (12S-rRNA), 16S ribosomal DNA (16S-rRNA) and cytochrome b (cytB). Primers can be found in Table S1.1. To improve taxonomic coverage, our sequence dataset was supplemented with sequences from Van Dooren, Thomassen, Smit, Helmstetter, and Savolainen (2018). When sequences from different sources were combined, the sequences were of the same source population (Table S1.2). Sequences were aligned in GENEIOUS (v6.1, Biomatters) using the MAFFT alignment plugin (v7.017; Katoh, Misawa, Kuma, & Miyata, 2002). Poorly aligned ends were trimmed.

2.2 | Phylogenetic inference

Sequences representing 63 *Austrolebias* individuals and two outgroups were used to build nDNA and mtDNA-based trees and a tree

constructed using nDNA and assuming a multispecies coalescent (*BEAST). Divergence times were estimated using secondary calibrations from a tree of the order Cyprinodontiformes (Helmstetter et al., 2016). Phylogenetic tree inference was conducted using BEAST2 v2.4.3 (Bouckaert et al., 2014) and led to three separate maximum clade credibility trees. Further details on our tree inference approach, including divergence time calibration, can be found in Appendix S2.

For downstream analyses we converted mtDNA and nDNA trees to species trees with the GLASS algorithm (Liu, Yu, & Pearl, 2009) using the speciesTree function in R library 'ape' (Paradis, Claude, & Strimmer, 2004). This created a pair of species trees we could use along the *BEAST species tree for further analyses.

2.3 | Phylogenetic clustering

We tested for the presence of phylogenetic clustering or overdispersion in the assemblages of each of the four areas of endemism. Initially we calculated phylogenetic diversity (PD) for all assemblages. We then calculated mean pairwise phylogenetic distance (MPD) and mean nearest taxon distance (MNTD; Webb et al., 2002), simulating null distributions for these statistics per assemblage (*ses.mntd* and *ses.mpd* in R package 'picante'; Kembel et al., 2010). Using the same package and the 'MicEco' package (Russel, 2019) we also calculated phylogenetic beta diversity with the functions *ses.comdist* & *ses.comdistnt* (999 randomisations) to interpret the phylogenetic distances between assemblages.

2.4 | Environmental niches

We aggregated coordinates of ponds where *Austrolebias* have been observed using primary publications—the Global Biodiversity Information Facility (https://www.gbif.org/) and several other sources (see Table S1.3, updated until March 2017). The number of sites used for each species can be found in Table S1.4. We characterized the environmental niche of each species using variables available from curated databases and related to climate, to the height of points above the ground, soil composition and river basin characteristics at the known pond sites (see Table S1.5). Per species, we averaged standardized environmental variables over the locations where it was found and principal component analysis (PCA) transformed the outcomes into a set of traits that were not correlated (see supplementary methods for further details).

2.5 | Body size data

To prevent the comparative analyses from becoming dependent on sampled age variation and environmental variability, which cannot be standardized, we decided to focus on the maximum size possible for a species. Growth is indeterminate in fish and age information is often not available. Taking an average of size measurements and assigning it to each species would lead to underestimates because of the inclusion of younger fish, rather than our goal of a size that reflects maximum fitness for a species. Body size data were therefore obtained by taking the largest known field measurements of adult male standard length (SL) for each species from the literature and our own field records (full dataset in Table S1.6).

2.6 | Estimation of ancestral ranges

Ancestral range estimation (ARE) was conducted using the R package 'BioGeoBEARS' (Matzke, 2014). 'BioGeoBEARS' fits the Dispersal-Extinction-Cladogenesis (DEC) model (Ree & Smith, 2008) and models similar to the BayArea (Bayesian Inference of Historical Biogeography for Discrete Areas) model (Landis, Matzke, Moore, & Huelsenbeck, 2013) and the Dispersal–Vicariance (referred to as DIVA) model (Ronquist, 1997), with and without founder-event speciation (indicated by +j). We used areas of endemism (Costa, 2010) for a broad scale representation of species range. These were: the western region of the Paraguay River basin (Western Paraguay or W), the lower La Plata River basin and the middle-lower Uruguay River basin (La Plata or L), the Negro River drainage of the Uruguay River basin and the upper/middle parts of the Jacuí, Santa Maria, Jahuarão and Quaraí river drainages (Negro River or N) and the Patos-Merin lagoon system including the southern coastal plains (Patos Lagoon or P; Figure 1). The middle section of the Iguaçu River basin is home to a single species, *Austrolebias carvalhoi*, for which we do not have data, so it was not included.

We ran separate sets of analyses where the maximum number of areas composing ancestral ranges was restricted to two (maximum observed) or four (maximum possible). We ran two additional sets of analyses: first, an analysis where ranges were restricted to include only adjacent areas. Second, we tested the hypothesis of Costa (2010) by implementing connections in the model corresponding to marine transgressions in the middle and late Miocene (15–11 Ma). We performed model selection based on Akaike information criterion corrected for sample size (AICc), to assess model fit. We then implemented Biogeographical Stochastic Mapping (BSM; Dupin et al., 2016) to estimate the frequencies of different biogeographic events (500 simulations).

2.7 | Selection regime shifts for size and environmental niche shifts

We used SURFACE (Ingram & Mahler, 2013) and l1OU (Khabbazian, Kriebel, Rohe, & Ané, 2016) to fit OU models with one or several optima and apply adapted information criterion (IC) in stepwise model comparisons. We identified selection regime shifts in body size and environmental niche shifts and then tested whether shifts converged towards the same optimum. Both methods are known to overestimate the number of shifts when relying on IC alone (Bastide, Ané, Robin, & Mariadassou, 2018; Khabbazian et al., 2016). We therefore performed

simulations to test if we could reject null models of no regime shifts: an OU model with just a single optimum or a BM model (Cooper, Thomas, Venditti, Meade, & Freckleton, 2016). Univariate (size) or multivariate (environmental niche traits) BM and OU models with a single optimum were fitted to trees and trait data. Using a parametric bootstrap, datasets were resampled according to the fitted OU and BM models and the SURFACE/l1OU model selection routine was applied to each of these resampled datasets. We then compared the IC differences for the actual data with quantiles of the distributions of IC from the simulations. When actual IC differences were larger than the 95% quantile of IC change, we rejected the model on which the simulations were based.

As there were more environmental variables than species in this study, we restricted the amount of environmental niche traits for which we tried to detect shifts using a non-phylogenetic PCA on the niche traits was carried out. We then limited our analysis of regime shifts to the first two principal components (PCs) of environmental variables. However, it is known that carrying out (phylogenetic) PC transformations can affect a subsequent analysis (Adams & Collyer, 2018; Bastide et al., 2018), so when we detected a regime shift in a PC, we tried to confirm the result for the niche trait contributing most to that PC.

3 | RESULTS

3.1 | Phylogenetic inference

The topologies of nDNA (Figure 2a) and mtDNA (Figure 2c) trees were generally well-supported with posterior probabilities (PP) > 0.9 in 67% and 69% of nodes respectively. Support in the coalescent tree (Figure 2b) was markedly lower (38% of nodes with PP > 0.9, 58% of nodes with PP > 0.75). Estimates of the crown age were between 8.47 and 36.52 Ma across our three trees, similar to what was inferred (10–15 Ma) in a recent study of the Cynolebiini (Costa, Amorim, & Mattos, 2017). We consistently recovered three major groups within *Austrolebias* in all trees (Figure 2).

Austrolebias bellottii and *Austrolebias apaii* were treated as the same species because *A. apaii* is a junior synonym of *A. bellottii* (García et al., 2012). We also decided to merge *Austrolebias vazferreirai* and *Austrolebias cinereus* for the comparative analyses in this paper, including the *BEAST analysis, in agreement with a recent proposal to consider *A. vazferreirai* a junior synonym of *A. cinereus* (Loureiro et al., 2018). Here, we refer to both as *A. vazferreirai*, as most individuals included were originally assigned to that species. Our mtDNA topology differed extensively from our nDNA tree (Figure 2), which had substantial effects on downstream inference. There are many reasons why the mtDNA history may not accurately reflect the species tree e.g. mitochondrial capture and lineage sorting (Ballard & Whitlock, 2004). Similarly, the poor support in the coalescent tree means that it is likely unreliable for inference. Therefore we focus on summarizing the results based on the nDNA tree.

3.2 | Phylogenetic clustering

Phylogenetic diversity was highest in Patos Lagoon and lowest in Western Paraguay (Table S1.7). We found consistently significant

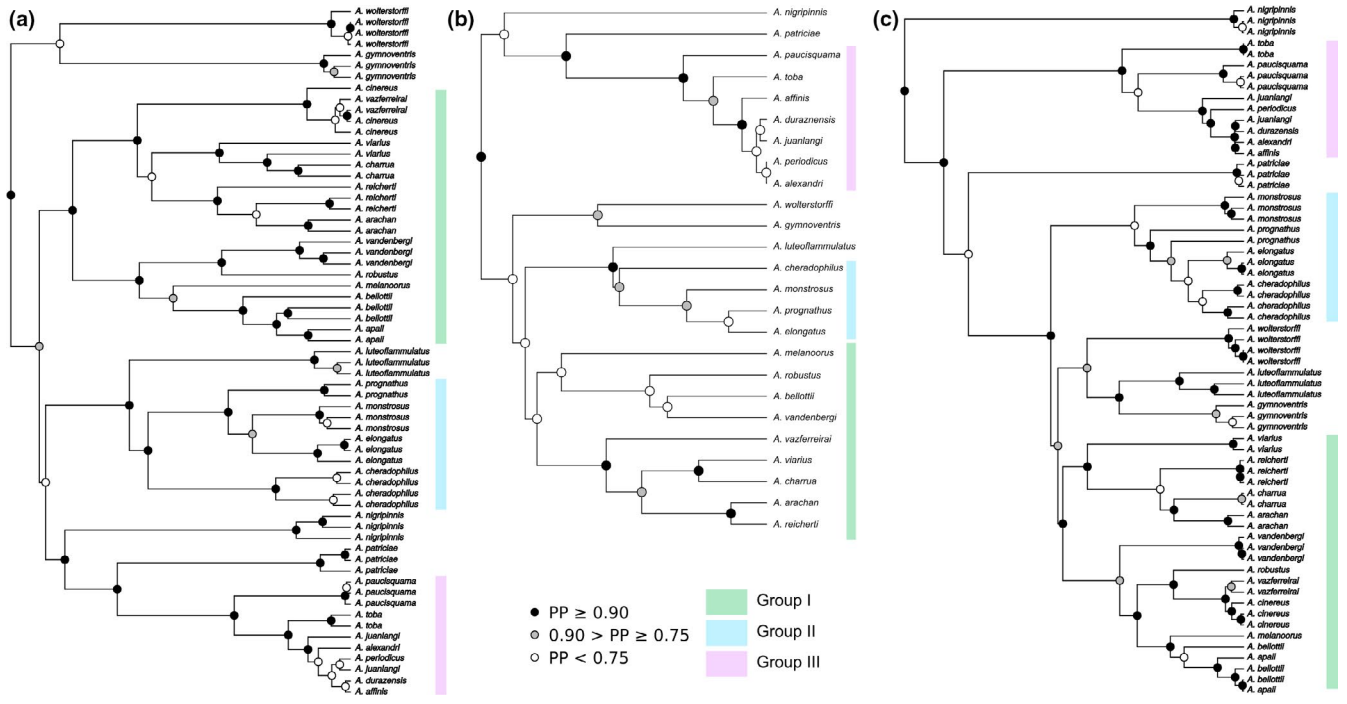

FIGURE 2 Maximum clade credibility trees for *Austrolebias* from Bayesian analyses of (a) nDNA, (b) coalescent nDNA and (c) mtDNA. Black circles indicate a posterior probability (PP) from 0.90 to 1.00 and grey circles indicate a PP from 0.75 to 0.90. PP < 0.75 is depicted as a white node. Highlighted, colour-coded sections represent three major groups that are recovered in all trees. Relationships within groups vary among trees

phylogenetic clustering for the Negro River assemblage using MPD and MNTD (Table S1.7). For the other assemblages, results using the nDNA tree revealed no significant patterns. We examined the relatedness of assemblages using phylogenetic beta diversity. We found a trend for Western Paraguay and River Negro to be closer to each other than expected based on MPD ($z = -1.42$, $p = 0.085$) but this was not the case for MNTD ($z = -0.93$, $p = 0.19$).

3.3 | Ancestral range estimation

The best fitting ARE models were DIVA + j for all trees. The parameters and likelihoods of all models assessed are summarized in Table S1.8. Models taking account of adjacency and marine transgressions did not significantly improve the likelihood (AICc difference < 2) when compared to the set of simpler models (Table S1.8). There is therefore no support for the hypothesis that the pattern of transgressions during the Middle and Late Miocene determined dispersal. Models with the jump dispersal parameter conferred significantly higher likelihood to the data (Table S1.9) and this event type was common (Figure 3; Figure S1.1), making up more than 50% of events in most cases (Figure S1.2). One striking result was that Western Paraguay was colonized by *Austrolebias* in four (nDNA) independent instances (Figure 3). Estimated numbers of within-area speciation events were particularly high in Patos Lagoon (an average of 4.6 events per simulation) and Negro River (2.9) while the other areas had averages below one. Vicariance events were rare, probably due to the rarity of current and ancestral ranges with more than one area.

The jump dispersal parameter has been recently criticized (Ree & Sanmartín, 2018 but see Klaus & Matzke, 2020) in regard to the overestimation of the number of jump dispersal events. Therefore, we also present results from the best fitting models without jump dispersal (DIVA in all cases; Figure S1.3) though the results generally provide the same interpretation.

3.4 | Size evolution

Our ancestral state reconstructions (Figure 4a) show substantial divergence in size. The four largest *Austrolebias* species form a single clade. Models consistently found a shift in selection optimum towards this clade (Group II in Figure 2) in all trees, just after the divergence of the smaller *Austrolebias luteoflammulatus*. (Figure 4a; Figure S1.4; SURFACE AICc decrease relative to single optimum model nDNA: 13.2, mtDNA:15.4, coalescent: 11.1). The SURFACE 5% quantile for the AICc decreases obtained from simulations of the BM null hypothesis is 11.8 for the nDNA tree. Likewise, for an OU model with a single optimum as null hypothesis, the 5% quantile equals 11.9. The results obtained using l1OU and based on phylogenetic Bayesian Information Criteria (pBIC) were concordant (nDNA) both for the shifts (Figure S1.5) and in having a pBIC difference exceeding the 5% quantile of the simulations. Therefore, a BM model or an OU model with a single optimum for size in the genus is implausible. We found no evidence for convergence between different selection regime shifts for size.

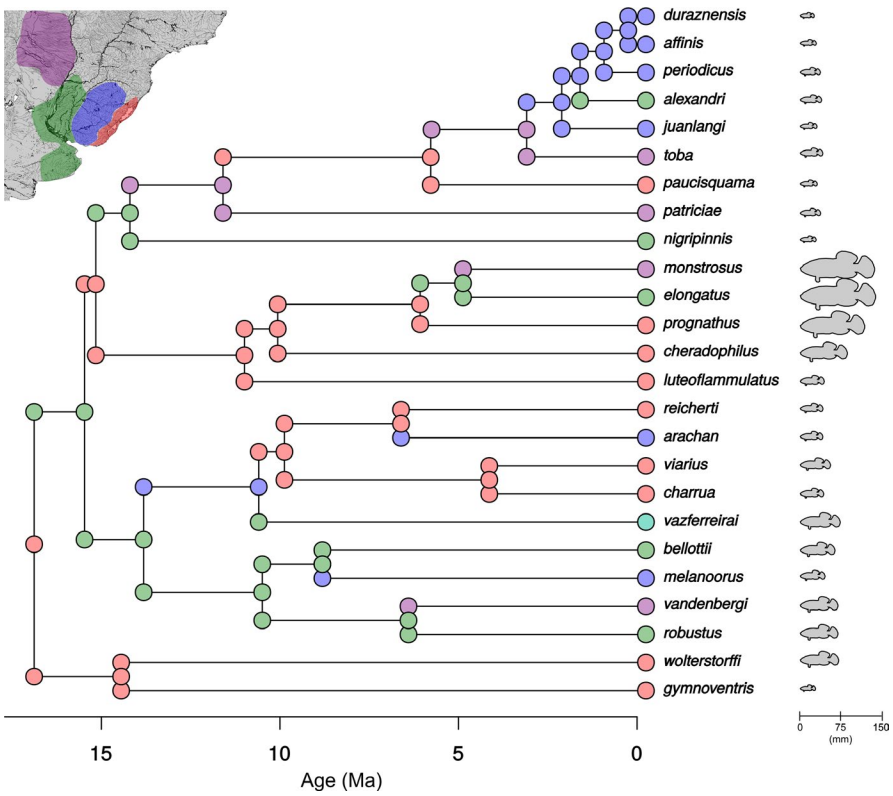

FIGURE 3 A biogeographic estimation of ancestral ranges for *Austrolebias* using the trimmed nDNA tree and the best fitting model (DIVA + j with a maximum of two areas) in BioGeoBEARS. Most probable states are shown on each node and corner of the tree, current ranges on the tips. Colours correspond to regions as depicted in the inset map in the top left and Figure 1. The current range of *Austrolebias vazferreirai* consists of two areas—Negro River and La Plata (coloured as teal). The body lengths of each species are shown as silhouettes to scale right of the tree

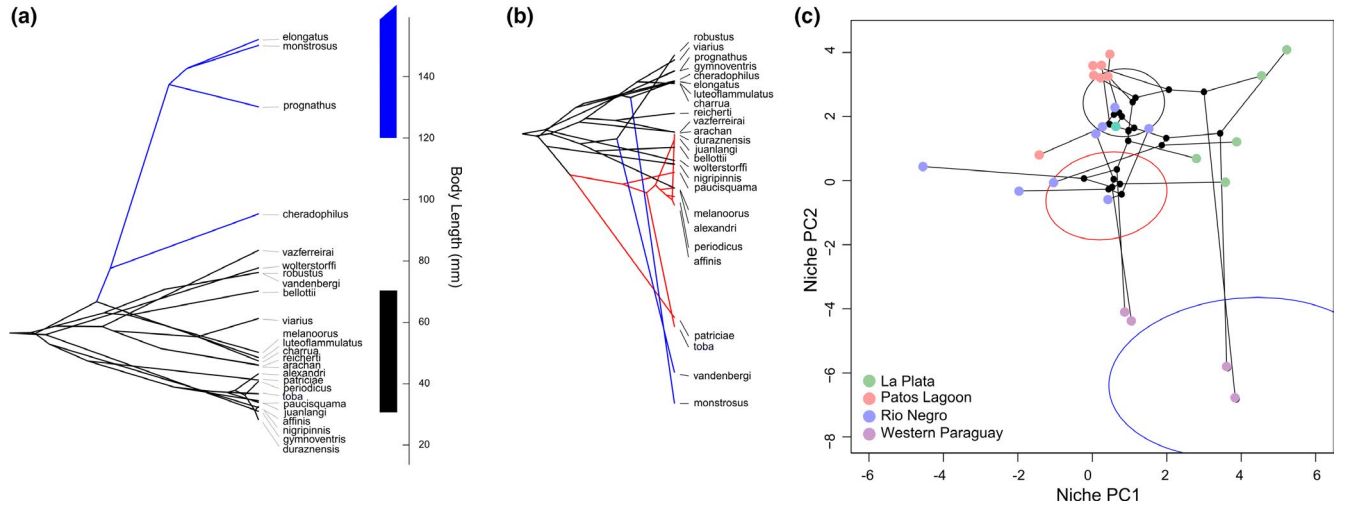

FIGURE 4 (a) Ancestral state reconstruction of body size across *Austrolebias* using stabletraits (Elliot & Mooers, 2014) and the nDNA tree. Ancestral values shown are the medians of the posterior distributions of size. Colours correspond to the selective optima species are tending towards as inferred by SURFACE. Bars on the right show the 95% confidence intervals of these optima. The interval for the larger size (blue) is truncated. (b) The nDNA tree arranged by the species values for principal component two (PC2) of the environmental niche. Coloured branches show the clades in which niche shifts have taken place. (c) A scatterplot of the species averages of the first two principal components. Points are tips and nodes of the nDNA tree, connected by branches representing the inferred topology. Tip labels are circles coloured based on current areas of endemism as shown in Figure 1. Black points represent median ancestral values reconstructed from the bivariate species averages using stabletraits. The 95% confidence ellipses for the combination of environmental niche values characterizing each regime estimated by SURFACE are shown in their respective colours

3.5 | Environmental niches

The first two PCs of the environmental niche variables explained 60% of species variation. Species phenotypically cluster to some degree by area of endemism (Figure 4b,c) and along two axes. Patos Lagoon and Western Paraguay species occupy two extremes for PC2, and all other species and ancestors group along an axis mostly determined by PC1. PC1 consists largely of soil information and temperature seasonality, plus information on the driest and coldest season. Western Paraguay and La Plata are similar for this PC and have less acidic soil with more silt, a larger seasonal range in temperatures and less annual precipitation. PC2 contains information relating to average temperature, precipitation seasonality and precipitation/temperature during the wettest month and the warmest season. This axis primarily distinguishes Western Paraguay from the other areas, with warmer temperatures, increased precipitation seasonality and more rain in the warmest season.

SURFACE analyses using PC1 & PC2 revealed three regime shifts in environmental niche, of which two (towards *Austrolebias monstrosus* and *Austrolebias vandenbergi*) converge to the same optimum trait values (Figure 4; Figure S1.4; AICc differences nDNA: 13.0 mtDNA: 11.5 coalescent: 12.9). The third tends towards an intermediate value between the background optimum and that of *A. vandenbergi* and *A. monstrosus*. Simulations allowed us to reject the hypothesis of a single environmental niche optimum for the genus for the nDNA tree (5% quantile bivariate OU model: 10.6). The bivariate BM model cannot be rejected using SURFACE (5% quantile nDNA: 17.1), but the result on the actual data corresponds to the 10% quantile, which still indicates that a model with multiple optima is likely a better fit. When using l1OU, both the BM and OU models are rejected (selected nDNA model pBIC 19.3, 5% quantile BM model: 8.3 OU model: 8.8). l1OU analyses find the three shifts for the nDNA tree, and infer shifts on the mtDNA and coalescent trees for *A. monstrosus* and *A. vandenbergi* (Figure S1.6). However, the main decrease in AICc and pBIC occurred when the model selection procedures explored the possibility of convergence and made the optimum trait values of the shifts for *A. vandenbergi* and *A. monstrosus* equal. It did not occur when the shift towards the intermediate optimum was added, and this might therefore be non-significant.

Differences among optima were always larger in PC2 and smaller in PC1. When we investigated whether shifts occurred for mean annual temperature (the variable with the largest weight on PC2) we found the same patterns. When comparing these shifts to ARE results we find that at least two and perhaps all four species from Western Paraguay were subject to shifts associated with their dispersal into the area (Figure 4b,c). Convergent shifts towards a new and shared environmental niche occurred independently when *A. vandenbergi* and *A. monstrosus* colonized Western Paraguay. None of the regime shifts for environmental niche are associated with selection regime shifts for size.

4 | DISCUSSION

Using models of trait evolution beyond BM and assessing them in the context provided by historical biogeography allowed us to identify that, despite the apparent similarity of *Austrolebias* assemblages, each followed different ecological and evolutionary trajectories.

4.1 | Evidence for phylogenetic clustering in one assemblage

Traditional community phylogenetic approaches concluded that only one of the four assemblages deviated from null expectations. The Negro River assemblage was significantly more phylogenetically clustered than expected. However, there is no support for a selection regime shift increasing phenotypic variation in body size within this area. Environmental niches of species in Negro River were not strongly phenotypically clustered (Figure 4c) and we found no robust evidence for a niche regime shift here. Therefore an adaptive radiation or environmental filtering cannot be inferred from the phylogenetic clustering. Alternatively, we can explain it by the relatively recent diversification of several species within the assemblage, after marine incursions in the late Miocene (Del Rio, Martínez, McArthur, Thirlwall, & Pérez, 2018).

4.2 | A single selection regime shift for body size

The processes acting in a single area of endemism have been critical for generating size variation across the genus. Our selection regime analyses consistently recovered a single body size shift towards the largest species. We found that this shift occurred in Patos Lagoon just after *A. luteoflammulatus* (Figures 3 and 4a) diverged. *A. luteoflammulatus* is a typical small species, *Austrolebias cheradophilus* is a large omnivore and *Austrolebias prognathus* is a very large piscivore. We speculate that a change in diet (see Laufer et al., 2009; Ortiz & Arim, 2016) may have been associated with the regime shift for size, and that predation by a piscivore facilitates coexistence of competitors.

A study of 48 species in the comparable African annual killifish genus *Nothobranchius* showed that most assemblages showed little variation in body size and only a single group of coexisting species exhibited size differences that may have been the result of ecological character displacement (Lambert, Reichard, & Pincheira-Donoso, 2019). In the La Plata and Patos Lagoon areas small, similar-sized *Austrolebias* species co-occur (Figure 1), along with the larger species. This is unexpected if competition between similar-sized species is strong among all annual fish in the same assemblage. Furthermore, *Austrolebias* can be found in ponds together with other small annual killifishes such as *Trigonectes aplocheiloides*, *Papiliolebias bitteri* and *Neofundulus paraguayensis* (Alonso, Calviño, Terán, & García, 2016) in Western Paraguay, and with *Cynopoecilus melanotaenia* (Canavero et al., 2014) in Patos Lagoon. These other species may have played a role in how species assemblages were constructed in some areas—by preventing the establishment of additional small *Austrolebias* species in Western Paraguay, for example.

We note that factors affecting differently sized species could similarly affect size variation within species. In phylogenetic comparative methods, within-species trait variation is seen as a source of uncertainty in species traits (Garamszegi, 2014) and thus difficult to account for in a meaningful way. Intraspecific variability in annual killifish is limited to some extent by synchronous hatching when ponds become wet (García, Loureiro, Machín, & Reichard, 2018), and interspecific competitors seem to cause little change to growth curves of a focal species (García, Smith, Machín, Loureiro, & Reichard, 2019).

4.3 | Convergent shifts for environmental niche

Environmental niches of species from Western Paraguay were markedly differentiated from the others. SURFACE analyses revealed that the colonization of Western Paraguay coincided twice with substantial and convergent shifts towards new environmental niches—potentially instrumental in the construction of the Western Paraguay assemblage. A characteristic that distinguishes Western Paraguay from other assemblages is that ponds fill in winter (Schalk, Montaña, Winemiller, & Fitzgerald, 2016) rather than in summer (Errea & Danulat, 2001) and this could explain the niche shifts in these seasonal species. Our results show that dispersal into particular areas can be mediated by environmental differences that must be overcome by colonizing species, regardless of size, thus having an effect on how assemblages are formed. However, as the environmental niche can vary without the need for evolutionary change we acknowledge that we cannot be certain that niche changes associated with dispersal involved selection regime shifts or adaptation.

A striking contrast to the environmental niche differentiation we demonstrated in South American killifish is the pattern in assemblages of five species of the genus *Nothobranchius*, which showed only moderate environmental niche differences (Reichard, Janáč, Polačik, Blažek, & Vrtílek, 2017). Altitude was found to be the most important factor explaining the distribution of different clades and species belonging to this African group. Environmental niche differences in *Austrolebias* stemmed from differences in soil characteristics, temperature and precipitation, indicating that different factors can govern geographic distributions of different annual fish clades despite their life cycle similarities.

4.4 | Repeated patterns are the result of different processes

The evolution of size variation can be explained by a specific process in three of the four assemblages. We observed a single selection regime shift in size, indicating that there was a single source of major size variation in the genus. Historical biogeographic approaches estimated that the speciation event that preceded this shift took place in the Patos Lagoon area. The species involved in this shift, (*A. cheradophilus*, *A. luteoflammulatus* and *A. prognathus*) currently coexist within the same ponds (Figure 1), which lends support to the idea that they coexisted during divergence. It is difficult to determine exactly what kind of interaction was behind size divergence but it may be that competition or cannibalism led to the emergence of large predator species (Van Dooren et al., 2018). Therefore, Patos Lagoon represents one extreme of size redistribution by dispersal because major size

variation originated within the area. The Patos Lagoon area has apparently been less affected by marine incursions in the Miocene (Hubert & Renno, 2006), which may have limited immigration of species from other areas and thus prolonged the time interval where within-area speciation could fill available niches (Emerson & Gillespie, 2008).

Our best fitting ARE model (Figure 3) revealed two major events that were key in distributing size variation across assemblages. First, a jump dispersal (or vicarance; Figure S1.3) event around 6 Ma into La Plata led to *Austrolebias elongatus*, followed by an event into Western Paraguay which led to *A. monstrosus*. Costa (2010) suggested that marine transgressions during the Middle Miocene may have connected Western Paraguay, Negro River and Patos Lagoon. We did not find support for the hypothesis that these marine transgressions in particular determined the pattern of dispersal events in the genus. Many important dispersal events occurred later, in particular arrivals in Western Paraguay and Negro River and the two dispersal events of piscivorous species above. This is in agreement with a recent dating of incursions in the Late Miocene (Del Rio et al., 2018).

A. vazferreirai (*cinereus*) is the largest species in the Negro River assemblage and is thought to be a generalist (Costa, 2006) rather than a piscivore. Negro River is the youngest assemblage, so there may not have been enough time for a large predator to colonize. Loureiro et al. (2018) discuss river capture events suggesting that these might have permitted dispersal between areas of endemism. However, within the Negro River catchment, drainage rearrangements might also have created locally isolated populations and provoked the relatively recent within-area speciation events.

4.5 | Perspective

Understanding the processes that lead to current species assemblages is central in the study of ecology and evolution (Hutchinson, 1959), and our work adds to increasing knowledge of the importance of species interactions (Cornell & Lawton, 1992) and size differences (Simberloff & Boecklen, 1981). We find similarities between our results and those from other study groups. Leitão et al. (2016) showed, using fish, trees and birds, how rare species had a disproportionate role in the functional structure of assemblages. This may also be the case in *Austrolebias*, where large species are typically rarer than smaller species (Lanés, Gonçalves, & Volcan, 2014) and occupy different functional niches, therefore widening the functional richness of assemblages. Analyses of the co-occurrence of related species of hummingbirds highlighted the importance of multiple mechanisms including competition, dispersal and the environment, in shaping assemblages (Weinstein, Graham, & Parra, 2017). Although the methodological approaches were different, we also found that these mechanisms, as well as historical biogeography, were likely important in generating assemblages of *Austrolebias*.

The differences between assemblages suggest two specific follow-up studies. We have found niche trait selection regime shifts for species in the Western Paraguay area, but that does not demonstrate adaptation to the local environmental conditions. The species from the La Plata area only differ in a single niche PC and lab or transplant studies using species from these areas could provide support for the hypothesis that they are locally adapted. The Negro River assemblage has no local piscivore, because it has not arrived there yet or because of local conditions. There are sites without piscivores in the nearby Patos Lagoon area as well. Studies on selection pressures on body size in these areas can help determine which factors contribute to the establishment and maintenance of piscivores.

5 | CONCLUSION

We have demonstrated that the simultaneous inspection of reconstructed trait evolution and historical biogeography can provide a useful perspective on ecological processes operating in assemblages. A single selection regime shift towards large body size was critical for generating the similar trait distributions observed in *Austrolebias* assemblages. It occurred during a series of speciation events in a single assemblage. Historical biogeographic reconstructions revealed that divergence in allopatry redistributed major size variation to other areas and that an environmentally distinct area was repeatedly colonised. Despite their apparent similarities, *Austrolebias* assemblage compositions and histories vary greatly. Our approach will be useful for other studies examining the processes that shape trait-structured assemblages.

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DATA AVAILABILITY STATEMENT

Tree and alignment files can be found on Dryad (https://doi.org/10.5061/dryad.zcrjdfn7v) and sequences generated for this study were uploaded to Genbank (accession no. KT94613-KT946590).

ORCID

Andrew J. Helmstetter https://orcid.org/0000-0003-3761-4981
Alexander S. T. Papadopulos https://orcid.org/0000-0001-6589-754X
Javier Igea https://orcid.org/0000-0001-9493-2076
Tom J. M. Van Dooren https://orcid.org/0000-0003-1137-9995

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BIOSKETCH

Andrew J. Helmstetter is interested in using phylogenetic and population genetic approaches to understand the origins of diversity. He asks questions related to the distribution of diversity within species, among geographic areas and across the tree of life. His research focuses on a variety of study groups from fish to crops to rain forest trees.

Author contributions: AJH, ASTP and TVD designed the study. AJH and TVD performed analyses with assistance from JI and ASTP. AJH and TVD wrote the manuscript with input from ASTP and JI.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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